

**Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol**

**Supplemental Assay Method for the Detection of
Extraneous Cytopathogenic Agents in Master Seed Virus**

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Standard Requirement: 9 CFR, Parts 113.46 and 113.55

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Supplemental Assay Method for the Detection of Extraneous Cytopathogenic Agents in
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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) detects extraneous cytopathogenic agents in master seed viruses (MSV) used in the production of veterinary vaccines. Cytopathic effect (CPE) of extraneous agents is determined by microscopic examination and May-Grünwald-Giemsa staining of tissue culture monolayers.

1.2 Keywords

Master Seed, CPE, *in vitro*, viral

2. Materials

2.1 Equipment/instrumentation

2.1.1 Inverted light microscope¹

2.2 Reagents/supplies

2.2.1 Cell culture flask,² 25 cm², of cellular monolayers meeting the requirement of the Code of Federal Regulations, Title 9 (9 CFR), Parts 113.51 and 113.52

2.2.1.1 Monolayers at least 7 days from last subculture

2.2.1.2 1 flask for each cell type to be inoculated with MSV (MSV Flasks)

2.2.1.3 1 flask for each cell type to be used as uninoculated negative control (NC Flasks)

2.2.2 Deionized water (DW)

2.2.3 0.01 M phosphate buffered saline (PBS)

¹Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent
²Cat. No. 430168, Corning Costar Corp., 45 Nagog Park, Acton, MA 01720 or equivalent

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2.2.3.1 1.33 g sodium phosphate, dibasic,
anhydrous³ (Na_2HPO_4)

2.2.3.2 0.22 g sodium phosphate, monobasic,
monohydrate⁴ ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

2.2.3.3 8.5 g sodium chloride⁵ (NaCl)

2.2.3.4 Q.S. to 1000 ml with DW.

2.2.3.5 Adjust pH to 7.2-7.6 with 0.1 N sodium
hydroxide⁶ (NaOH) or 1.0 N hydrochloric acid⁷
(HCl).

2.2.4 Absolute Methanol⁸

2.2.5 May-Grünwald Stain⁹

2.2.6 Whatman[®] No. 2 filter paper¹⁰

2.2.7 Giemsa Stain¹¹

2.2.8 Pipettes: 1 ml,¹² 5 ml,¹³ 10 ml,¹⁴ and 25 ml¹⁵

2.2.9 Graduated cylinder, 500 ml¹⁶

2.2.10 Mineral oil¹⁷

³ Cat. No. S 0876, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

⁴ Cat. No. S 9638, Sigma Chemical Co. or equivalent

⁵ Cat. No. S 9625, Sigma Chemical Co. or equivalent

⁶ Cat. No. 925-30, Sigma Chemical Co. or equivalent

⁷ Cat. No. 920-1, Sigma Chemical Co. or equivalent

⁸ Cat. No. A412-500, Fisher Scientific Corp., 1600 West Glenlake Avenue, Itasca, IL 60143 or equivalent

⁹ Cat. No. 21661, Para Scientific Co., Cedar Lane and Tyburn Road, Fairless Hills, PA 19030 or equivalent

¹⁰ Cat. No. 28455-030, VMR Scientific Products Corp., 1430 Waukegan Road, McGaw Park, IL 60085 or equivalent

¹¹ Cat. No. SG28-100D, Fisher Scientific Corp., or equivalent

¹² Falcon[®] 7521, Fisher Scientific Corp. or equivalent

¹³ Falcon[®] 7543, Fisher Scientific Corp. or equivalent

¹⁴ Falcon[®] 7504, Fisher Scientific Corp. or equivalent

¹⁵ Falcon[®] 7525, Fisher Scientific Corp. or equivalent

¹⁶ Cat. No. P-34546-06, Cole-Parmer Instrument Co., 625 Bunker Court, Vernon Hills, IL 60061-9872 or equivalent

¹⁷ Cat. No. 0121-1, Fisher Scientific Corp. or equivalent

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3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture, MSV extraneous agent testing as described in the 9 CFR, Parts 113.55 and 113.46, cell staining techniques, and use of inverted light microscopes.

3.2 Preparation of reagents/control procedures

3.2.1 May-Grünwald Stain, Working Dilution

3.2.1.1 Using a graduated cylinder, measure 500 ml of Absolute Methanol. The Absolute Methanol shall be at room temperature (RT), $23^{\circ} \pm 2^{\circ}\text{C}$, prior to use.

3.2.1.2 Dissolve 0.5 g May-Grünwald Stain in Section 3.2.1.1.

3.2.1.3 Filter Section 3.2.1.2 using a No. 2 filter.

3.2.1.4 Store at RT.

3.2.2 Giemsa Stain, Working Dilution

3.2.2.1 Using a 25-ml pipette, measure 24 ml DW into a suitable container. Using a 1-ml pipette, add 1 ml of Giemsa Stain, or dilute as specified by the manufacturer.

3.2.2.2 Store at RT.

3.3 Preparation of the sample

MSV shall be tested as described in the 9 CFR, Part 113.55. The last subculture shall meet the minimum requirements specified in the 9 CFR, Part 113.46. Monolayers shall be microscopically examined regularly for evidence of CPE during the 14-day maintenance period. Monolayers at least

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7 days from the last subculture shall be tested for extraneous CPE agents by the May-Grünwald-Giemsa staining procedure, using at least 1 MSV Flask and 1 NC Flask for each cell type. Just prior to staining, all flasks are examined for CPE.

4. Performance of the test

CAUTION: Precautionary measures for using methanol and Giemsa stain: keep away from heat, spark, or flames; keep container closed; avoid breathing vapor; use with adequate ventilation; wear appropriate protective clothing including gloves and safety goggles (no contact lenses); wash thoroughly after handling. Store in flammable liquid storage cabinet. Place used chemicals in appropriate containers for disposal in a manner consistent with safe laboratory practices.

4.1 May-Grünwald-Giemsa Staining

4.1.1 Decant tissue culture media from the MSV Flasks and NC Flasks.

4.1.2 Using a 10-ml pipette, rinse monolayers with 10 ml of RT PBS.

4.1.3 Decant the PBS rinse.

4.1.4 Using a 5-ml pipette, add 4 ± 1 ml of the Working Dilution May-Grünwald Stain into each flask.

4.1.5 Incubate at RT for 10 ± 5 min.

4.1.6 Decant the stain and dispose of it properly.

4.1.7 Using a 5-ml pipette, add 3 ml of the Working Dilution Giemsa Stain into each flask.

4.1.8 Incubate at RT for 15 ± 5 min.

4.1.9 Decant the stain and dispose of it properly.

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4.1.10 Using a 10-ml pipette, rinse monolayers twice with 10 ml of DW.

4.1.11 Decant the DW.

4.1.12 While monolayers are still moist, using an inverted microscope at 100-200X compare the MSV Flasks to the NC Flasks of the corresponding cell line tested. Mineral oil, 2 ml, may be placed in each flask to allow examination at a later time.

4.1.12.1 Examine each flask for the presence of extraneous intracellular agents. The May-Grünwald-Giemsa differentially stains DNA and RNA nucleoproteins. DNA nucleoprotein stains red-purple while RNA nucleoprotein stains blue.

4.1.12.2 Examine for the presence of CPE, such as inclusion bodies, giant cell formation, syncytia, or other cell abnormalities which may be attributable to an extraneous agent.

5. Interpretation of the test results

5.1 Criteria for a valid test

5.1.1 The NC Flask is free of extraneous intracellular agents, CPE, or cellular abnormalities.

5.1.2 The NC Flask and the MSV Flask are free of bacterial or fungal contamination.

5.1.3 The MSV Flask is free of CPE attributable to the MSV agent.

5.1.4 If any of the validity criteria in **Sections 5.1.1 through 5.1.3** are not met, the test is a **NO TEST** and is repeated without prejudice.

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5.2 Satisfactory tests

If the test is valid and the MSV Flask is found free of extraneous intracellular agents, CPE, or cellular abnormalities, the MSV is **SATISFACTORY**.

5.3 Retests

5.3.1 If the initial test is valid and extraneous intracellular agents, CPE, or cellular abnormalities are found, the test is repeated (1st retest), using only the cell type found positive. The test is repeated using a new vial of MSV.

5.3.2 If the second valid test (1st retest) confirms the initial result, the MSV is **UNSATISFACTORY**.

5.3.3 If the second valid test (1st retest) fails to confirm the initial result, the MSV is tested a third time (2nd retest). The test is repeated using a new vial of MSV.

5.3.3.1 If the second and third valid tests (1st and 2nd retests) result in the MSV Flasks being free of extraneous intracellular agents, CPE, or cellular abnormalities, the MSV is **SATISFACTORY**.

5.3.3.2 If the third valid test (2nd retest) confirms the initial result, the MSV is **UNSATISFACTORY**.

6. Report of test results

The results are appropriately recorded and reported on a data sheet.

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7. References

7.1 Code of Federal Regulations, Title 9, Parts 113.46, 113.51, 113.52, and 113.55, U.S. Government Printing Office, Washington, DC, 1999.

7.2 Gurr, E., *Staining Practical and Theoretical*, Williams and Wilkins Co., Baltimore, MD, 1962, p. 220.

7.3 Carlson, F.L., *Histotechnology*, American Society of Clinical Pathologists, Chicago, IL, 1990, pp. 110-112.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous SAM 312, dated June 1, 1985.